REMARKS

The applicant thanks the Examiner for granting a telephonic interview regarding the present application on January 25th, 2007.

Reconsideration of the rejections set forth in the Office action mailed March 21, 2006 is respectfully requested. Claims 1-10 are pending in the application. Claims 1-6 are currently withdrawn from consideration, and claims 7-10 are under examination.

I. Amendments

Claim 7 has been amended:

to recite that the pooled nucleic acid of step (a) is pooled genomic nucleic acid from a population of individuals (see, for example, page 6, lines 1-2);

to recite that the recognition site and cleavage site of the first restriction endonuclease are coextensive (see page 9, lines 24-25), and that digestion with the first restriction endonuclease of step (a) produces a first mixture of restriction fragments having first cleavage ends with predictable protruding strands (see page 13, lines 9-10);

to recite that the Exo III resistant linker of step (b) comprises at one terminus a protruding strand which hybridizes to that of said first cleavage ends (see page 13, lines 19-20) and further comprises, at its opposite terminus, a 3'-overhang (see page 20, lines 6-8, and Fig. 12, step 3, which shows the attached linkers with terminal 3'-overhangs);

to recite that the recognition site and cleavage site of the second restriction endonuclease are coextensive (see page 9, lines 24-25), and that the second restriction endonuclease of step (c) is different from the first restriction endonuclease, and is selected such that the frequency of its restriction sites in the pooled genomic nucleic acid is less than that of the restriction sites of the first restriction endonuclease (see page 14, lines 1-4), and to recite that fragments produced by cleavage with the second restriction endonuclease of step (c) have a protruding strand (see page 14, line 2); and

to recite that ligation of the Exo III susceptible linker of step (d) results in each fragment produced by cleavage with said second restriction endonuclease bearing an Exo III susceptible terminus, selected from the 5'-overhang or the blunt end of the linker (see Fig. 12, steps 3-4, which shows these fragments with blunt ended linkers attached).

Dependent claim 8 has been amended to recite that contacting the reannealed third ligation product of step (g) (claim 7) with a single strand dependent nuclease is effective to digest mismatched duplexes (see page 20, lines 23-24).

The specification has been amended to add a reference number (234) present in Figure 2B.

No new matter is added by any of the amendments.

II. Information Disclosure Statement

The Examiner stated that a non-patent reference was missing from the Information Disclosure Statement submitted March 11, 2003. A copy of this reference (Lisitsyn *et al.*, *Science* **259**(5097):946-951, 1993) is enclosed. (This article was cited as an "A" reference in the International Search Report, indicating that it was considered "a document defining the general state of the art which is not considered to be of particular relevance.")

III. Rejections under 35 U.S.C. §112, First Paragraph

Claims 7-10 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention.

The independent claim has been amended to more particularly define the materials employed in the claimed method; support in the specification for these amendments is noted in section I above.

The independent claim has been amended to more particularly define the starting nucleic acid (pooled genomic nucleic acid from a population of individuals). The sequences per se of the pooled genomic nucleic acid need not be known beforehand and are not critical to the execution of the method.

The independent claim has also been amended to more particularly define the restriction endonucleases used. The endonucleases produce fragments with protruding strands, not blunt ended fragments. The claims also recite that the recognition site and cleavage site of the endonucleases are coextensive (as recited in the specification at page 9, lines 24-25). Accordingly, an endonuclease having a cleavage site separate from its recognition site, such as a type IIS endonuclease, would not be used. The claims also recite that the frequency of cleavage

sites for the first endonuclease in the target nucleic acid is greater than that for the second endonuclease (see page 14, lines 1-4).

The independent claim has also been amended to more particularly define the linkers which are ligated to the protruding strands produced by enzymatic cleavage. The linkers, recited in steps (b) and (d) of claim 7, have, in addition to a structural feature which renders them Exo III resistant or susceptible, respectively, a protruding strand which hybridizes to the protruding strands of the respective fragment cleavage ends. Accordingly, blunt ended ligation is not employed, and the linkers are designed such that their protruding strands will hybridize with those of the cleaved fragments. As shown in the drawings (e.g. Figure 12), the linkers are designed such that this structural feature (blunt end or overhang) is on the end of the linker that is not attached to the fragment.

As stated in the MPEP at §2163(I)(A) (page 2100-167), "There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed.... the issue of a lack of adequate written description may arise even for an original claim when an aspect of the claimed invention has not been described with sufficient particularity such that one skilled in the art would recognize that the applicant had possession of the claimed invention. The claimed invention as a whole may not be adequately described if the claims require an essential or critical feature which is not adequately described in the specification and which is not conventional in the art or known to one of ordinary skill in the art."

In the present case, applicant submits that all aspects and elements of the claimed method have been described with sufficient particularly in the specification (with particular reference to pages 13-14, 19-21, and Figure 12) that one skilled in the art would recognized that applicant had possession of the claimed method, and that no essential or critical feature of the claimed method that is "not conventional in the art" lacks adequate description in the specification.

In view of the foregoing, the elements of the claimed method are fully described in the specification. Applicant submits that claims 7-10 comply with the requirements of 35 U.S.C. §112, first paragraph.

IV. Rejections under 35 U.S.C. §102(e)

Independent claim 7 was rejected under 35 U.S.C. §102(b) as being anticipated by Short *et al.*, U.S. Patent No. 6,352,842. This rejection is respectfully traversed for the following reasons.

A. The Claimed Method

Independent claim 7 is directed to a method of making a reference library comprising a mixture of heterogeneous nucleic acid fragments which has been enriched for polymorphic sequences. The method comprises the following sequence of steps. It is clear that the steps are carried out in the indicated order, as each of steps (b)-(g) recites (as indicated in **boldface**) an intermediate product produced in the previous step (indicated in *italics*).

(a) digesting pooled genomic nucleic acid from a population of individuals with a first restriction endonuclease, whose recognition site and cleavage site are coextensive, to produce a first mixture of *restriction fragments* having first cleavage ends with predictable protruding strands;

(b) ligating an Exo III resistant linker to the first cleavage ends of **said restriction fragments**, to form a *first ligation product population*,

wherein said Exo III resistant linker comprises at one terminus a protruding strand which hybridizes to that of said first cleavage ends, and further comprises, at its opposite terminus, a 3'-overhang;

(c) digesting **said first ligation product population** with a second restriction endonuclease, whose recognition site and cleavage site are coextensive, wherein the second restriction endonuclease is different from the first restriction endonuclease, and is selected such that the frequency of its restriction sites in the pooled genomic nucleic acid is less than that of the restriction sites of the first restriction endonuclease;

to form a second mixture of restriction fragments, wherein those fragments produced by cleavage with said second restriction endonuclease have a *second cleavage end*, having a protruding strand;

(d) ligating an Exo III susceptible linker, comprising either a 5'-overhang or a blunt end, and to **each said second cleavage end**, such that each said fragment produced by cleavage with said second restriction endonuclease bears an Exo III susceptible terminus selected from said 5'-overhang and said blunt end,

thereby forming *a second ligation product population*,

wherein said Exo III susceptible linker comprises a first member of a binding pair;

(e) digesting **said second ligation product population** with Exo III to form *a third ligation product population*, comprising (i) single stranded DNA comprising end sequences corresponding to said Exo III resistant and Exo III susceptible linkers and (ii) double stranded DNA comprising end sequences corresponding to said Exo III resistant linkers;

(f) denaturing **said third ligation product population** and hybridizing the mixture so obtained to form *a reannealed third ligation product population*; and

(g) contacting **said reannealed third ligation product population** with a second member of said binding pair to isolate duplexes containing said Exo III susceptible linker, thereby to enrich for duplexes which form a polymorphic reference population of restriction fragments.

Brief Summary of the Process

A summary of the process, and an annotated copy of Figure 12 illustrating the process, were submitted with the faxed materials forwarded on January 23, 2007. To briefly recapitulate this summary: Of the fragments making up the "second ligation product population" of step (d) (shown at (4) in Fig. 12), only those which were cleaved at least once by the second restriction endonuclease will have an "Exo III susceptible linker" attached. Thus, when the fragment population is treated with Exo III, fragments which had the second restriction site are either digested completely (bottom fragment in (4), having two Exo III susceptible linkers) or rendered single stranded (middle fragment in (4), having one Exo III susceptible linker). The fragments which did not have the second restriction site (top fragments in (4)) have two Exo III resistant linkers and are not affected by Exo III.

The single stranded fragments (which, again, had the second restriction site) are then hybridized with one strand of their counterparts which did not contain the second restriction site (top fragment in (4)), hence giving duplexes which are polymorphic at the location of the second restriction site (shown in step 6 in Fig. 12).)

B. The Prior Art

The Short *et al.* patent teaches (see Field of the Invention) a "directed evolution method for preparing a polynucleotides encoding polypeptide [sic], which method comprises the step of generating site-directed mutagenesis optionally in combination with the step of polynucleotide chimerization, the step of selecting for potentially desirable progeny molecules, including by a

process termed end-selection (which may then be screened further), and the step of screening the polynucleotides for the production of polypeptide(s) having a useful property".

The Short *et al.* patent is not concerned with locating or isolating polymorphic sequences in pooled nucleic acids, which is the object of the currently claimed invention.

The Examiner points to different locations in the specification of Short *et al.* that allegedly describe steps such as: digesting nucleic acids with restriction enzymes; producing Exo III resistant or susceptible linkers; ligation; denaturing and hybridization of nucleic acids; and binding and enriching of nucleic acids.

For example, the Examiner refers to column 37, line 9 to column 39, line 35 of Short *et al.* as teaching the use of Exo III digestion and of Exo III-resistant or -susceptible "linkers". A portion of this disclosure is reproduced below (emphasis added; different typeface used for clarity). (The remaining portions of column 37, line 9 to column 39, line 35 of Short *et al.* contain no reference or only very general reference to exonuclease III.)

> Exonuclease-mediated Shuffling
>
> In a particular embodiment, this invention provides for a method for shuffling, assembling, reassembling, recombining, &/or concatenating at least two polynucleotides to form a progeny polynucleotide (e.g. a chimeric progeny polynucleotide that can be expressed to produce a polypeptide or a gene pathway). In a particular embodiment, a double stranded polynucleotide end (e.g. two single stranded sequences hybridized to each other as hybridization partners) is treated with an exonuclease to liberate nucleotides from one of the two strands, leaving the remaining strand free of its original partner so that, if desired, the remaining strand may be used to achieve hybridization to another partner.
>
> . . .
>
> The exonuclease action of exonuclease III requires a working double stranded polynucleotide end that is either blunt or has a 5' overhang, and the exonuclease action is comprised of enzymatically liberating 3' terminal nucleotides, leaving a single stranded 5' end that becomes longer and longer as the exonuclease action proceeds (see FIG. 1). Any 5' overhangs produced by this approach may be used to hybridize to another single stranded polynucleotide sequence (which may also be a

single stranded polynucleotide or a terminal overhang of a partially double stranded polynucleotide) that shares enough homology to allow hybridization. The ability of these exonuclease III-generated single stranded sequences (e.g. in 5' overhangs) to hybridize to other single stranded sequences allows two or more polynucleotides to be shuffled, assembled, reassembled, &/or concatenated.

Furthermore, it is appreciated that one can protect the end of a double stranded polynucleotide or render it susceptible to a desired enzymatic action of a serviceable exonuclease as necessary. For example, a double stranded polynucleotide end having a 3' overhang is not susceptible to the exonuclease action of exonuclease III. **However, it may be rendered susceptible to the exonuclease action of exonuclease III by a variety of means; for example, it may be blunted by treatment with a polymerase, cleaved to provide a blunt end or a 5' overhang, joined (ligated or hybridized) to another double stranded polynucleotide to provide a blunt end or a 5' overhang, hybridized to a single stranded polynucleotide to provide a blunt end or a 5' overhang, or modified by any of a variety of means).**

According to one aspect, an exonuclease may be allowed to act on one or on both ends of a linear double stranded polynucleotide and proceed to completion, to near completion, or to partial completion. When the exonuclease action is allowed to go to completion, the result will be that the length of each 5' overhang will be extend far towards the middle region of the polynucleotide in the direction of what might be considered a "rendezvous point" (which may be somewhere near the polynucleotide midpoint). Ultimately, this results in the production of single stranded polynucleotides (that can become dissociated) that are each about half the length of the original double stranded polynucleotide (see FIG. 1). Alternatively, an exonuclease-mediated reaction can be terminated before proceeding to completion.

Thus this exonuclease-mediated approach is serviceable for shuffling, assembling &/or reassembling, recombining, and concatenating polynucleotide building blocks, which polynucleotide building blocks can be up to ten bases long or tens of bases long or hundreds of bases long or thousands of bases long or tens of thousands of bases long or hundreds of thousands of bases long or millions of bases long or even longer.

This exonuclease-mediated approach is based on the action of double

stranded DNA specific exodeoxyribonuclease activity of E. coli **exonuclease III. Substrates for exonuclease III may be generated by subjecting a double stranded polynucleotide to fragmentation.** Fragmentation may be achieved by mechanical means (e.g., shearing, sonication, etc.), by enzymatic means (e.g. using **restriction enzymes**), and by any combination thereof. Fragments of a larger polynucleotide may also be generated by polymerase-mediated synthesis.

Exonuclease III is a 28K monomeric enzyme, product of the xthA gene of E. coli with four known activities: exodeoxyribonuclease (alternatively referred to as exonuclease herein), RNaseH, DNA-3'-phosphatase, and AP endonuclease. The exodeoxyribonuclease activity is specific for double stranded DNA. The mechanism of action is thought to involve enzymatic hydrolysis of DNA from a 3' end progressively towards a 5' direction, with formation of nucleoside 5'-phosphates and a residual single strand. The enzyme does not display efficient hydrolysis of single stranded DNA, single-stranded RNA, or double-stranded RNA; however it degrades RNA in an DNA-RNA hybrid releasing nucleoside 5'phosphates. The enzyme also releases inorganic phosphate specifically from 3'phosphomonoester groups on DNA, but not from RNA or short oligonucleotides. Removal of these groups converts the terminus into a primer for DNA polymerase action. . . .

Thus, the reference teaches generally that polynucleotides to be treated with Exo III may be fragmented with restriction endonucleases (col 39, lines 13-16; highlighted above). It also includes a general description of the use of Exo III for "shuffling" of polynucleotides, and it teaches that one may attach an Exo III susceptible linker to a polynucleotide, so that "it may be rendered susceptible to the exonuclease action of exonuclease III" (col 38, lines 45-51; highlighted above).

However, nowhere does the reference teach attaching an Exo III resistant linker, comprising a 3'-overhang, to a polynucleotide, as recited in step (b) of applicant's claim 7.

Nor does the reference teach using Exo III to digest a "second ligation product population" as recited in step (e) of applicant's claim 7, where this "second ligation product population" is produced by:

(a) digesting pooled genomic nucleic acid from a population of individuals with a first restriction endonuclease...**to produce a first mixture of restriction fragments** having first cleavage ends with predictable protruding strands;

(b) ligating an Exo III resistant linker, comprising a 3'-overhang, to the first cleavage ends of **said restriction fragments**, to form **a first ligation product population**;

(c) digesting **said first ligation product population** with a second restriction endonuclease ... to form a second mixture of restriction fragments, wherein those fragments produced by cleavage with said second restriction endonuclease at least one **second cleavage end**, having a protruding strand; and

(d) ligating an Exo III susceptible linker, comprising a first member of a binding pair, to each **said second cleavage end**, to form **a second ligation product population**.

Furthermore, the reference does not teach:

(f) denaturing a third ligation product population, itself produced by Exo III treatment of such a "second ligation product population", and hybridizing the mixture so obtained to form a reannealed third ligation product population; and

(g) contacting said reannealed third ligation product population with a second member of said binding pair to isolate duplexes containing said Exo III susceptible linker, thereby to enrich for duplexes which form a polymorphic reference population of restriction fragments.

The Examiner also refers to column 9, line 66 to column 10, line 11 of Short *et al.*, and to column 12, lines 4-16 (both reproduced below) for teaching of ligating Exo III resistant or Exo III susceptible linkers.

> "Directional ligation" refers to a ligation in which a 5' end and a 3' end of a polynucleotide are different enough to specify a preferred ligation orientation. For example, an otherwise untreated and undigested PCR product that has two blunt ends will typically not have a preferred ligation orientation when ligated into a cloning vector digested to produce blunt ends in its multiple cloning site; thus, directional ligation will typically not be displayed under these circumstances. In contrast, directional ligation will typically displayed when a digested PCR product having a 5' EcoR I--treated end and a 3' BamH I--is ligated into a cloning vector that has a multiple

cloning site digested with EcoR I and BamH I. (column 9, line 66 to column 10, line 11)

The above passage appears to have little or no relevance to ligation of Exo III resistant or Exo III susceptible linkers.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Sambrook et al, 1982, p. 146; Sambrook, 1989). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

As used herein, "linker" or "spacer" refers to a molecule or group of molecules that connects two molecules, *such as a DNA binding protein and a random peptide*, and serves to place the two molecules in a preferred configuration, e.g., so that the random peptide can bind to a receptor with minimal steric hindrance from the DNA binding protein. (column 12, lines 4-16)

These very general definitions have no direct relevance to ligation of Exo III resistant or Exo III susceptible linkers.

For a prior art reference to anticipate in terms of 35 U.S.C. §102, every element of the claimed invention must be identically shown in a single reference. ...The elements must be arranged as in the claim under review... *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990). (MPEP §2131)

For the reasons outlined above, the Short *et al.* patent does not teach the method claimed by the applicant as embodied in steps (a)-(g) of independent claim 7. Accordingly, the applicant respectfully requests that this rejection under 35 U.S.C. §102(e) be withdrawn.

V. Further Rejections under 35 U.S.C. §102(e)

Independent claim 7 and dependent claims 8-9 were rejected under 35 U.S.C. §102(e) as being anticipated by Barany *et al.*, U.S. Patent No. 6,027,889. This rejection is respectfully traversed for the following reasons.

A. The Claims

Independent claim 7 is described above; dependent claims 8-9 include all of the limitations of claim 7.

B. The Prior Art

Barany *et al.* is directed to "the detection of nucleic acid sequence differences using coupled ligase detection reaction [LDR] and polymerase chain reaction [PCR]" (Abstract; Summary).

The methods employed in Barany *et al.* detect sequence differences (e.g. SNPs) via sequence-specific hybridization of probes along a template, followed by ligation. When the probes do not hybridize completely (i.e. when there is a sequence mismatch), no ligation occurs. (See, for example, the description at column 21, lines 11-39, with reference to Fig. 4.) This approach is entirely different from that of the currently claimed method, which detects polymorphisms at restriction sites, using a process employing digestion of pooled nucleic acid by different restriction endonucleases.

Similar to the treatment of Short *et al.*, above, the Examiner points to different locations in the specification of Barany *et al.* that allegedly describe steps such as: digesting genomic DNA with restriction enzymes; employing Exo III resistant or susceptible linkers; denaturing and hybridization of nucleic acids; and contacting nucleic acids to a solid support (which in Barany *et al.* refers to an array of probe sequences).

For example, the Examiner refers to column 26, lines 6-36 of Barany *et al.* as teaching the use of Exo III digestion and of Exo III-resistant or -susceptible "linkers". This disclosure is reproduced below (emphasis added; different typeface used for clarity).

> FIG. 12 is a schematic drawing showing a solution to the allele specific LDR/PCR problem, utilizing an intermediate exonuclease digestion step. Allele-specific LDR/PCR can be achieved while significantly reducing background ligation independent (incorrect) target amplification. To do so, it is necessary to eliminate one or more of the components required for ligation independent PCR amplification, without removing the information content of the ligation product sequence.
> One solution is to use exonuclease in step 2 to digest unreacted LDR oligonucleotide probes from step 1. By blocking the end which is not

ligated, for example the 3' end of the downstream oligonucleotide probe, one probe can be made substantially resistant to digestion, while the other is sensitive. Only the presence of full length ligation product sequence will prevent digestion of the upstream primer. **Blocking groups include use of a thiophosphate group and/or use of 2-O-methyl ribose sugar groups in the backbone.** Exonucleases include Exo I (3'-5'), Exo III (3'-5'), and Exo IV (both 5'-3' and 3'-5'), the later requiring blocking on both sides. One convenient way to block both probes is by using one long "padlock" probe (see M. Nilsson et. al., "Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection," Science 265: 2085-88 (1994), which is hereby incorporated by reference), although this is by no means required. An advantage of using exonucleases, for example a combination of Exo I (single strand specific) and **Exo III (double strand specific)**, is the ability to **destroy both target and one LDR probe**, while leaving the ligation product sequences substantially undigested. By using an exonuclease treatment prior to PCR, in accordance with steps 3 and 4, either one or both oligonucleotide probes in each set are substantially reduced, and thus hybridization of the remaining oligonucleotide probes to the **original target DNA (which is also substantially reduced by exonuclease treatment)** and formation of a ligation product sequence which is a suitable substrate for PCR amplification by the oligonucleotide primer set is substantially reduced. In other words, formation of ligation independent labeled extension products is substantially reduced or eliminated.

The reference thus describes the use of "blocking groups", such as thiophosphate groups, to prevent digestion by exonucleases. However, the reference does not teach "ligating an Exo III susceptible linker", as recited in step (d) of applicant's independent claim, to any polynucleotide.

In fact, the reference does not teach "ligating an Exo III resistant linker" which "comprises at one terminus a protruding strand which hybridizes to that of said first cleavage ends, and further comprises, at its opposite terminus, a 3'-overhang", as recited in step (b) of applicant's independent claim. The only exonuclease "blocking groups" taught in Barany are "a thiophosphate group and/or use of 2-O-methyl ribose sugar groups in the backbone" of the polynucleotide.

The only specifically described use of Exo III in the above passage refers to digestion of the

"original target DNA", which is double stranded. There is no reference to any blocking groups (or "Exo III resistant linkers") applied to this target, since the goal is to "substantially reduce" the amount of original target DNA, as stated at column 26, lines 22-34: "An advantage of using exonucleases, for example a combination of Exo I (single strand specific) and **Exo III** (double strand specific), is the ability to destroy both **target** and one LDR probe, while leaving the ligation product sequences substantially undigested. By using an exonuclease treatment prior to PCR, in accordance with steps 3 and 4, either one or both oligonucleotide probes in each set are substantially reduced, and thus hybridization of the remaining oligonucleotide probes to the **original target DNA (which is also substantially reduced by exonuclease treatment)**. . .is substantially reduced.".

Finally, as stated for Short *et al.* above, nowhere does Barany *et al.* teach using Exo III to digest a "second ligation product population" as recited in step (e) of applicant's claim 7, where this "second ligation product population" is produced by:

(a) digesting pooled genomic nucleic acid from a population of individuals with a first restriction endonuclease...**to produce a first mixture of restriction fragments** having first cleavage ends with predictable protruding strands;

(b) ligating an Exo III resistant linker, comprising a 3'-overhang, to the first cleavage ends of **said restriction fragments**, to form **a first ligation product population**;

(c) digesting **said first ligation product population** with a second restriction endonuclease ... to form a second mixture of restriction fragments, wherein those fragments produced by cleavage with said second restriction endonuclease at least one **second cleavage end**, having a protruding strand; and

(d) ligating an Exo III susceptible linker, comprising a first member of a binding pair, to each **said second cleavage end**, to form **a second ligation product population**.

Furthermore, the reference does not teach:

(f) denaturing a third ligation product population, itself produced by Exo III treatment of such a "second ligation product population", and hybridizing the mixture so obtained to form a reannealed third ligation product population; and

(g) contacting said reannealed third ligation product population with a second member of

said binding pair to isolate duplexes containing said Exo III susceptible linker, thereby to enrich for duplexes which form a polymorphic reference population of restriction fragments.

The Examiner also refers to column 40, lines 18-46 (reproduced below) and to Table 11 in Barany *et al.* for teaching of ligating Exo III resistant or Exo III susceptible linkers.

> FIG. 24 shows the design of LDR oligonucleotide probes for quantification of gene amplifications and deletions in the LDR/PCR process. These oligonucleotide probes were designed to recognize exon 8 in the p53 tumor suppressor gene (on chromosome 17p), exon 3 of int-2 (on chromosome 11q), an internal exon in HER-2/neu (i.e. HER-2/neu/erbB oncogene) (on chromosome 17q), exon 3 in SOD (i.e. super oxide dimutase) (on chromosome 21q), and exon 6 in G6PD (i.e. glucose 6-phosphate dehydrogenase) (on chromosome Xq). Each pair of LDR oligonucleotide probes has the following features: (i) The left oligonucleotide probe contains from 5' to 3' an 18 base sequence identical to the fluorescently labeled secondary oligonucleotide primer (black bar), an "adjustment sequence" (white bar), and a target-specific sequence of from 22 to 28 bases with a Tm of 75°C (patterned bar); (ii) The right oligonucleotide probe contains from 5' to 3' a target-specific sequence of 20-25 bases with a Tm of 75°C (patterned bar), a single HaeIII or HinP1I restriction site at slightly different positions within the target-specific sequence, and an "adjustment sequence" (white bars). The two oligonucleotide probes are designed such that their combined length is exactly 96 bases, with 50 G+C bases and 46 A+T bases. The position of each unique restriction site generates a product which differs by at least 2 bases from the other products. Each oligonucleotide probe set has an exon-specific region chosen to ligate the junction sequence of (A, T)C↓C(A, T). This junction sequence corresponds to either a proline residue (codon CCN) or the complementary sequence of a tryptophan residue (TGG). These sequences were chosen to minimize differences in ligation rates and the chance of a polymorphism at the ligation junction.

Table 11 shows a list of primer sequences.

Neither the above passage nor the Table appears to have any relevance to ligation of Exo III

resistant or Exo III susceptible linkers.

For a prior art reference to anticipate in terms of 35 U.S.C. §102, every element of the claimed invention must be identically shown in a single reference. ...The elements must be arranged as in the claim under review... *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990). (MPEP §2131)

In view of the above, the Barany *et al.* patent in no way teaches the method claimed by the applicant. Accordingly, the applicant respectfully requests that this rejection the rejection under 35 U.S.C. §102(e) be withdrawn.

VI. Rejections under 35 U.S.C. §103(a)

Claims 7-10 were rejected under 35 U.S.C. §103(a) as being unpatentable over Short *et al.*, cited above, and Strathmann, U.S. Patent No. 6,480,791. The rejections are respectfully traversed in light of the following remarks.

A. The Claims

Independent claim 7 is described above; dependent claims 8-10 contain all the limitations of claim 7.

B. The Cited Art

As stated above, the disclosure of Short *et al.* describes various processes used in manipulations of nucleic acids, including restriction enzyme digestion, ligation, hybridization, etc., but it in no way teaches the method embodied in steps (a) – (g) of the applicant's independent claim.

As stated by the Examiner, Strathmann likewise teaches methods of nucleic acid amplification and sequencing, and it includes the use of single strand dependent nucleases and attachment of biotin to nucleic acids.

C. Analysis

Combining the "directed evolution" methods of Short *et al.* with the use of Exo I and biotin in Strathmann would not produce the method of applicant's claim 7. The disclosure of Strathmann does not make up for the numerous deficiencies of Short *et al.* with respect to the claimed method.

For example, neither reference teaches attaching an Exo III resistant linker, comprising a 3'-overhang, to a polynucleotide, as recited in step (b) of applicant's claim 7. Neither reference teaches using Exo III to digest a "second ligation product population" as recited in step (e) of applicant's claim 7, where this "second ligation product population" is produced by the process recited in steps (a)-(d) of claim 7.

In view of the foregoing, the applicant respectfully requests the Examiner to withdraw the rejection under 35 U.S.C. §103(a).

VII. Further Rejections under 35 U.S.C. §103(a)

Claims 7-10 were rejected under 35 U.S.C. §103(a) as being unpatentable over Barany *et al.*, U.S. Patent No. 6,027,889, cited above, and Barany *et al.*, U.S. Patent No. 6,534,293. The rejections are respectfully traversed in light of the following remarks.

A. The Claims

Independent claim 7 is described above; dependent claims 8-10 contain all the limitations of claim 7.

B. The Cited Art

As stated above, the disclosure of Barany *et al.* '889 does not teach the method embodied in steps (a) – (g) of the applicant's independent claim.

Like Barany *et al.* '889, U.S. Patent No. 6,534,293 describes methods of detecting sequence differences (e.g. SNPs) via sequence-specific hybridization of probes along a template, followed by ligation. When the probes do not hybridize completely (i.e. when there is a sequence mismatch), no ligation occurs. (See, for example, claim 1 of the patent.)

As stated by the Examiner, Barany *et al.* '293 also teaches linkers having biotin tags, in Tables 8-9 and 13. A representative such "linker" is "Biotin-C18 spacer- GAA TAC CCG GGA TGA CTA CGT", the first entry in Table 8. However, there is no teaching that any of these "linkers" is an "Exo III susceptible linker" as recited in applicant's claim 7, having either a 5'-overhang or a blunt end; or that it is attached to a "second cleavage end" produced by the process recited in steps (a)-(c) of applicant's claim 7.

The disclosure of Barany *et al.* '293 does not make up for the numerous deficiencies of Barany *et al.* '889 with respect to the claimed method.

For example, neither reference teaches attaching an Exo III resistant linker, comprising a 3'-overhang, to a polynucleotide, as recited in step (b) of applicant's claim 7. Neither reference teaches using Exo III to digest a "second ligation product population" as recited in step (e) of applicant's claim 7, where this "second ligation product population" is produced by the process recited in steps (a)-(d) of claim 7.

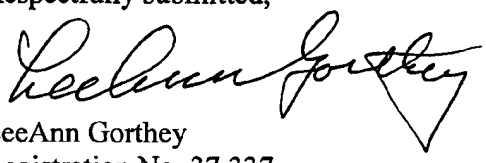
In view of the foregoing, the applicant respectfully requests the Examiner to withdraw the rejection under 35 U.S.C. §103(a).

VIII. Conclusion

In view of the foregoing, the applicant submits that the claims now pending are now in condition for allowance. A Notice of Allowance is, therefore, respectfully requested.

If in the opinion of the Examiner a further telephone consultation would expedite the allowance of the present application, the Examiner is encouraged to call the undersigned at (503) 727-2116.

Respectfully submitted,



LeeAnn Gorthey
Registration No. 37,337

Date: 2-15-07

Correspondence Address:
PAYOR NUMBER 22918
PHONE: (503) 727-2116
FAX: (650) 838-4350

Cloning the Differences Between Two Complex Genomes

Nikolai Lisitsyn, Natalya Lisitsyn, Michael Wigler

The analysis of the differences between two complex genomes holds promise for the discovery of infectious agents and probes useful for genetic studies. A system was developed in which subtractive and kinetic enrichment was used to purify restriction endonuclease fragments present in one population of DNA fragments but not in another. Application of this method to DNA populations of reduced complexity ("representations") resulted in the isolation of probes to viral genomes present as single copies in human DNA, and probes that detect polymorphisms between two individuals. In principle, this system, called representational difference analysis (RDA), may also be used for isolating probes linked to sites of genomic rearrangements, whether occurring spontaneously and resulting in genetic disorders or cancer, or programmed during differentiation and development.

Genetic alterations underlie various biological processes. Programmed gene rearrangements occur in many contexts, including the generation of diversity in the immune system (*1*), mating type switching in yeasts (*2*), and antigenic variation in microbial organisms (*3*). Spontaneous losses or rearrangements of genetic material in somatic cells occur frequently during the development of cancers and leukemias. Similar events occurring in the germ line can result in inherited disorders. Even infectious disease can be viewed as the alteration of the genetic content of the infected organism. Various time-consuming methods have been applied to determining the nature of the genetic changes that occur in the above situations. A single method for defining the differences between two DNA populations could, in principle, be applied to each of the above problems and lead to the discovery of the genetic basis of many types of biological phenomena. We present here a general method (representational difference analysis, or RDA) for finding small differences between the sequences of two DNA populations. The method builds upon subtractive hybridization techniques that have been used in the past to find probes for large sequence differences between two genomes.

In 1984 Lamar and Palmer applied a subtractive hybridization technique to clone probes for the Y chromosome (*4*). They used an excess of sheared DNA from a female to drive hybridization of Sau 3A–cleaved DNA from a male. The Y chromosome–specific DNA was free to self-anneal and was subsequently cloned after ligation into an acceptor site of a plasmid. A similar method was applied by Kunkel *et al.* (*5*) and then by Nussbaum *et al.* (*6*) to clone probes for the Duchenne muscular dystrophy and the choroidermia loci, respectively. In both cases, large deletions on the X chromosome made it possible to use subtractive hybridization techniques with DNA from affected males as "driver" and DNA from normals as "tester" (*7*). In general, however, subtractive hybridization techniques do not achieve sufficient enrichment of the sequences that occur only in tester (the "target") partly because of the high complexity of the human genome, which prevents effectively complete hybridization (*8*). Even when subtractive steps are reiterated, "target" sequences are enriched only 100 to 1000 times (*9*). This enrichment is insufficient for more common situations in which the magnitude of enrichment required is 10^6.

A second means for DNA difference enrichment has been proposed on the basis of the second-order kinetics of self-reassociation (*9*). In theory, this method can be applied after an initial enrichment of target sequences has been achieved by subtraction. If a population of DNA fragments containing a target subpopulation enriched n times relative to unenriched fragments in tester is melted and reannealed so that only a small proportion of double-stranded tester DNA forms, double-stranded target DNA would be present n^2 times relative to the other sequences present as duplex DNA (*10*). We call this "kinetic" enrichment. To apply this method, it is necessary to use some kind of DNA amplification to purify the small quantities of double-stranded DNA that form away from all driver and all unreannealed tester sequences. We achieved this by ligating oligonucleotide adaptors to tester so that only double-stranded tester molecules were amplified during the polymerase chain reaction (PCR) (*11*). Subsequent reiterations of the method led to exponentially increasing enrichments of target.

In RDA, we lowered the DNA complexity of both tester and driver genomes by preparing a representative portion of each genome (a "representation"). The difference analysis of the two representations is based on simultaneous combination of subtractive and kinetic steps. The lowered complexity of the representations allowed us to achieve greater completeness during subtractive enrichment and, hence, a more effective kinetic enrichment. We now demonstrate the cloning of probes for single copy sequences present in (or absent from) one of the two genomes, and the cloning of probes for binary polymorphisms between two individuals. We discuss the applications of this approach to the discovery of probes for pathogenic organisms, for otherwise anonymous loci that have suffered genetic rearrangements, and for polymorphisms located near the genes affected by inherited disorders.

Subtractive and kinetic enrichments of PCR amplicons. We began by making representations of DNA populations that we call "amplicons"; DNA cleaved with relatively infrequent cutting restriction endonucleases was ligated to oligonucleotide adaptors, and amplified by PCR. The result was similar to a size fractionation, since after 20 rounds of amplification only low molecular size fragments, below 1 kb, were effectively amplified. The advantage of this method for representation compared to a physical fractionation is that only small amounts of starting material (less than 10 μg) are required. Only a subset of the whole genome was represented. However, when different restriction endonucleases were chosen, sets of amplicons that scan the genome could be made. In the following examples, we made amplicons of mammalian DNA after it underwent cleavage with Bam HI, Bgl II, and Hind III (*12*). We estimate the complexity of the resulting amplicons to be 55 times, 13 times, and 8 times less than the complexity of the starting genomic DNA, respectively (*13*).

Once tester and driver amplicons were made, their adaptors were removed by cleavage, and only tester fragments were ligated to new adaptors at their 5′ ends. We then combined subtractive and kinetic steps into a single operation, the hybridization-amplification step (Fig. 1) (*14*). We abandoned all use of physical separation techniques, such as biotinylation with subsequent biotin-avidin chromatography (*9*, *15*) or hydroxylapatite (*9*), to enrich for double-stranded tester because these methods are not completely reliable. Instead, DNA amplification was used for selective enrichment of double-stranded tester. After melting and reannealing in the presence of excess of driver amplicon, DNA molecules were treated with Taq DNA polymerase at

The authors are at Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724.

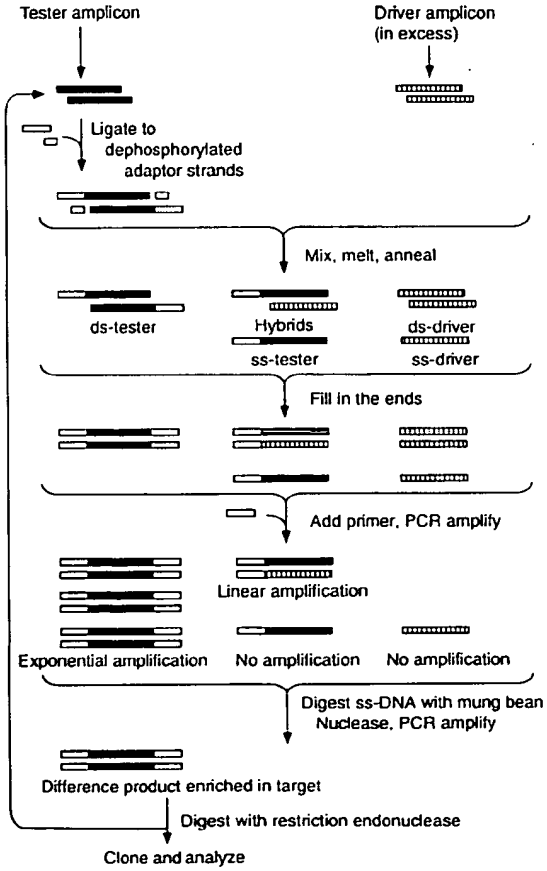
elevated temperatures in the presence of all four deoxynucleotide triphosphates. Only self-reannealed tester molecules had 5′ adaptors at each end of the duplex DNA and thus could be filled in at both 3′ ends. Therefore only self-reannealed tester could subsequently be amplified by PCR at an exponential rate. Driver DNA acted as a competitive inhibitor for the self-reannealing of those tester DNA fragments common to driver. Target DNA, which occurs only in tester, was thus enriched relative to other tester DNA after amplification. The PCR products were then either cloned, otherwise analyzed, or further enriched. In the last case, the PCR products were cleaved, and yet new PCR adaptors were added to their 5′ ends (*16*). This material was then diluted (*17*) with fresh driver amplicon and the hybridization-amplification step was repeated.

The first round of RDA was mainly subtractive. Subsequent rounds had a greatly increased kinetic component equal to the enrichment achieved on the previous step. For example, if target is equimolar with respect to tester (that is, single copy), and if driver amplicon is taken in n times excess to tester amplicon, target may be enriched e times after the first round, where $e < n$ (*8*). After the second round, target would be enriched yet another e times because of repetition of the subtractive component, multiplied by another e times because of the kinetic component. After the third round, total enrichment would be at least the square of that. If e is 50, at the end of the second round, target would be enriched by about 10^5, and at the end of the third round by more than 10^{10}.

Acquisition (or loss) of new sequences. In the first set of stringent tests of our protocols, we added, as single copies, adenovirus or bacteriophage λ DNA (or both) to a human DNA to create a model tester, and used the same DNA without viral DNA as driver. The viral DNA's were the target and we can view this model as testing the efficacy of the procedure in either of two situations: the acquisition of a pathogenic genome at a single copy of genomic DNA per infected cell; or the homozygous loss of DNA by spontaneous mutation. We prepared Bgl II amplicons from human DNA with adenovirus and λ DNA's as targets (Fig. 2A) or Hind III amplicons with λ DNA as target (Fig. 2B). If Bgl II amplicons were taken, small λ and adenovirus fragments were the major difference products even after two rounds (Fig. 2A, lane a). This represents an enrichment of more than 5×10^6 times from the starting material, and a probable enrichment of about 4×10^5 times from amplicons. There is a strong bias during amplification against fragments larger than 1.0 kbp, so that the larger Bgl II fragments of target (Fig. 2A, lanes c and d) are not enriched.

The enrichment from Hind III amplicons was not as effective. The λ Hind III fragment was greatly enriched after the third round (Fig. 2B, lane b), but other sequences were still present. Nevertheless, after the fourth round the expected target fragment was purified to near homogeneity (Fig. 2B, lane c). We attribute this outcome to the greater sequence complexity of the Hind III amplicon. In fact, we found that without some simplification of driver complexity, our method failed. Presumably, when the complexity of the driver was too high, subtractive and kinetic enrichments were diminished and competing processes dominated.

Probes for polymorphic loci. In the experiments described above, the driver and tester were identical except for the



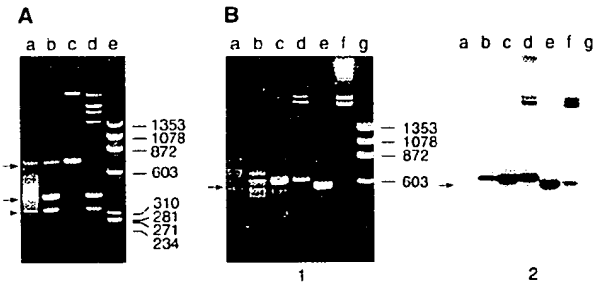


Fig. 1 (left). Schematic protocol for representational difference analysis, illustrating the hybridization and amplification steps after the preparation of amplicons. The representation stage is not illustrated. The details are described in (*12*). **Fig. 2 (above).** Representational difference analysis with viral DNA's added as targets. High molecular weight DNA (10 μg) purified from the lymphoid cell line DRL 484 was used for preparation of driver amplicons and 10 μg of the same DNA, containing equimolar amounts of target [120 pg of adenovirus-2 DNA or 160 pg of λ phage DNA (or both), both from New England Biolabs] was taken for preparation of tester amplicons (*12*). Difference products were prepared as described in (*14*). (**A**) Agarose gel electrophoresis of difference products of Bgl II amplicons, obtained after the second (lane a) and third (lane b) hybridization-amplification steps. λ phage (lane c) and adenovirus (lane d) amplicons were prepared as described (*12*) taking 1 to 5 ng of purified viral DNA ligated to adaptors (primer set 1; see Table 1). Hae III digest of ϕX174 RF DNA is in lane e. Sizes (bp) are indicated to the right. Arrows on the left indicate major difference products. (**B**) Agarose gel electrophoresis (B, 1) and Southern blot (B, 2) of difference products of Hind III amplicons. Difference products (0.5 μg) obtained after two, three, and four hybridization-amplification steps are shown in lanes a to c. The λ phage Hind III amplicons were prepared as described (*12*) taking 1 to 5 ng of purified viral DNA ligated to adaptor set 1 (lane d). The difference product after four hybridization-amplification steps was digested with Hind III (lane e). Hind III–digested λ phage DNA (lane f) and Hae III–digested ϕX174 DNA (lane g, sizes indicated to the right) are as shown. These DNA's were separated on a 2 percent agarose gel, transferred at reduced pressure with the LKB 2016 VacuGene Vacuum Blotting System (Pharmacia LKB Biotechnology) to GeneScreen Plus membranes (Du Pont) and hybridized to ^{32}P-labeled λ phage Hind III amplicon under conditions recommended by the membrane supplier. Arrow on left indicates the target λ fragment. The slight upward mobility shift of this fragment in lanes b, c, and d relative to lanes e and f is due to the presence of adaptors.

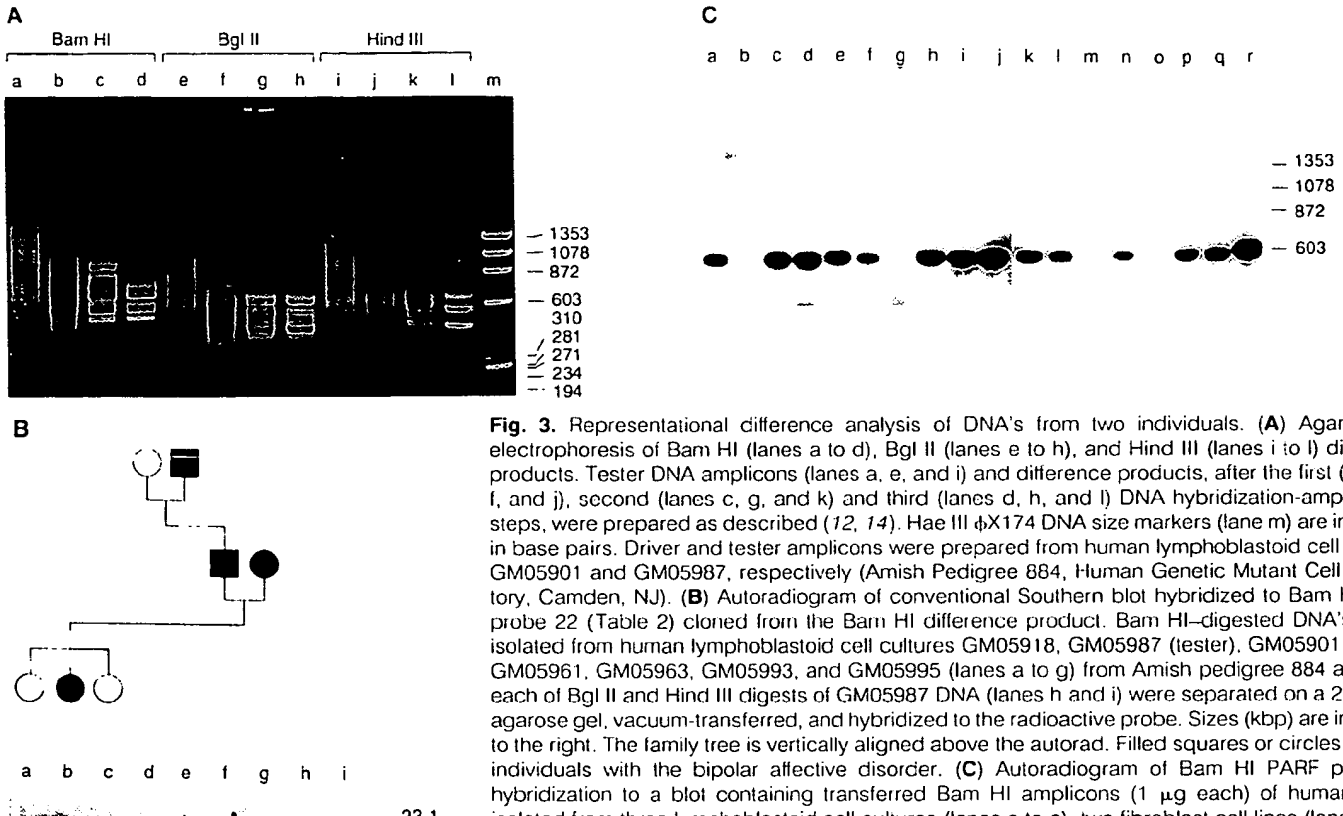


Fig. 3. Representational difference analysis of DNA's from two individuals. (**A**) Agarose gel electrophoresis of Bam HI (lanes a to d), Bgl II (lanes e to h), and Hind III (lanes i to l) difference products. Tester DNA amplicons (lanes a, e, and i) and difference products, after the first (lanes b, f, and j), second (lanes c, g, and k) and third (lanes d, h, and l) DNA hybridization-amplification steps, were prepared as described (*12, 14*). Hae III ϕX174 DNA size markers (lane m) are indicated in base pairs. Driver and tester amplicons were prepared from human lymphoblastoid cell cultures GM05901 and GM05987, respectively (Amish Pedigree 884, Human Genetic Mutant Cell Repository, Camden, NJ). (**B**) Autoradiogram of conventional Southern blot hybridized to Bam HI PARF probe 22 (Table 2) cloned from the Bam HI difference product. Bam HI–digested DNA's (2 μg) isolated from human lymphoblastoid cell cultures GM05918, GM05987 (tester), GM05901 (driver), GM05961, GM05963, GM05993, and GM05995 (lanes a to g) from Amish pedigree 884 and 2 μg each of Bgl II and Hind III digests of GM05987 DNA (lanes h and i) were separated on a 2 percent agarose gel, vacuum-transferred, and hybridized to the radioactive probe. Sizes (kbp) are indicated to the right. The family tree is vertically aligned above the autorad. Filled squares or circles indicate individuals with the bipolar affective disorder. (**C**) Autoradiogram of Bam HI PARF probe 22 hybridization to a blot containing transferred Bam HI amplicons (1 μg each) of human DNA's isolated from three lymphoblastoid cell cultures (lanes a to c), two fibroblast cell lines (lanes d and e), five human placentas (lanes f to j), and seven Amish pedigree lymphoblastoid cell cultures GM05918, GM05987, GM05901, GM05961, GM05963, GM05993, and GM05995 (lanes k to q). The Bam HI difference product (1 ng) after three hybridization amplification steps was placed in lane r. Sizes (in base pairs) are indicated to the right.

experimental perturbation introduced by the presence of exogenous DNA in tester. In order to assess the method for potential applications, we compared tester and driver amplicons from different individuals and expected to obtain a subset of restriction endonuclease fragments that were polymorphic between them. For example, if one of the two Bam HI sites flanking a short Bam HI fragment in tester was absent in both alleles from driver, leading to only large and poorly amplified Bam HI fragments in driver, the short Bam HI fragment of tester would be present in its Bam HI amplicon but absent in the Bam HI amplicon of the driver.

We were able to study two sisters from an Amish family with a well-established pedigree. Amplicons were prepared after cleavage with Bam HI, Bgl II, or Hind III. Difference products between amplicons were obtained (*12, 14*) and size-fractionated by gel electrophoresis (Fig. 3A). A discrete but complex pattern of bands was observed in each case. After three successive hybridizations and amplifications (Fig. 3A, lanes d, h, and l), difference products were cloned into plasmids. For each difference product we picked three probes for blot hybridization analysis, and found that all of them detected polymorphisms within the Amish family. We analyzed Bam HI difference products in the greatest detail (Table 2). Of 20 randomly picked clones, 12 different classes of clones remained after removing redundancies, and the inserts from nine of these were used as probes in DNA (Southern) blots of tester, driver, and five other members of the family. All probes detected two alleles, distinguished by a large and a small DNA fragment (Table 2, allele size). The small allele was always present in the tester (Fig. 3B, lane b, and Table 2, sample B) and always absent in the driver (Fig. 3B, lane c, and Table 2, sample A). The probes detected either one of these bands in presumed homozygotes (Fig. 3B, lanes c and e), or both bands in presumed heterozygous individuals (Fig. 3B, lanes a, b, d, f, and g). Moreover, the blot hybridization pattern for each probe was consistent with a Mendelian pattern of inheritance. From these experiments we concluded that our method yields collections of probes for restriction endonuclease fragment polymorphisms between two individuals.

Each of the Bam HI probes derived from the above experiment was also used in blot hybridizations to amplicons from the family and ten other unrelated human DNA's extracted from cell lines or placentas (Fig. 3C and Table 2). Such blots detect the presence or absence of small-size Bam HI fragments in the tested DNA, and thus are more readily performed than conventional Southern blots to total genomic DNA. We found that blotting amplicons was in complete concordance with blotting of total genomic DNA, as described above, in 63 out of 63 cases (nine probes times seven individuals). The results of the amplicon blots indicate that probes capable of detecting polymorphisms within the Amish family tend to detect polymorphisms in the human population at large. We call such polymorphisms PARF's, for polymorphic amplifiable restriction endonuclease fragments.

Table 1. Sequences of primers used for representational difference analysis. Primer set 1 (R series) was used for preparing amplicon representations, and sets 2 (J series) and 3 (N series) were used for odd and even hybridization–amplifications, respectively. The OLIGO computer program (National Biosciences) was used to check the oligonucleotide design for the absence of strong secondary structure.

Primer set	Name	Sequence
1	R Bgl24	5′-AGCACTCTCCAGCCTCTCACCGCA-3′
	R Bgl12	5′-GATCTGCGGTGA-3′
2	J Bgl24	5′-ACCGACGTCGACTATCCATGAACA-3′
	J Bgl12	5′-GATCTGTTCATG-3′
3	N Bgl24	5′-AGGCAACTGTGCTATCCGAGGGAA-3′
	N Bgl12	5′-GATCTTCCCTCG-3′
1	R Bam24	5′-AGCACTCTCCAGCCTCTCACCGAG-3′
	R Bam12	5′-GATCCTCGGTGA-3′
2	J Bam24	5′-ACCGACGTCGACTATCCATGAACG-3′
	J Bam12	5′-GATCCGTTCATG-3′
3	N Bam24	5′-AGGCAACTGTGCTATCCGAGGGAG-3′
	N Bam12	5′-GATCCTCCCTCG-3′
1	R Hind24	Same as R Bgl24 (see above)
	R Hind12	5′-AGCTTGCGGTGA-3′
2	J Hind24	Same as J Bgl24 (see above)
	J Hind12	5′-AGCTTGTTCATG-3′
3	N Hind24	5′-AGGCAGCTGTGGTATCGAGGGAGA-3′
	N Hind12	5′-AGCTTCTCCCTC-3′

Table 2. Screening for presence of Bam HI PARF's in 17 human DNA samples.

Probe		DNA sample*																Allele size (kbp)†	
No.	Percent‡	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	Large	Small
1	15.5	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15	0.61, 0.67§
11	14.4	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	15	0.6
6	8.9	−	+	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	3.5	0.58
19	5.5	−	+	+	−	+	+	+	+	+	+	−	+	−	−	+	+	+	15	0.51
17	4.4	−	+	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	8	0.48
22	4.4	−	+	+	+	−	+	+	+	−	+	+	+	+	−	+	+	+	6.5	0.67
8	3.3	−	+	+	+	−	+	+	+	+	+	+	−	−	−	+	+	+	ND	0.62
24	3.3	−	+	−	+	+	+	+	+	−	+	+	−	+	+	−	+	−	>50	0.65
26	3.3	−	+	+	−	−	−	+	+	+	+	−	+	−	+	+	+	−	6,5‖	0.65
9	2.2	−	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	ND	0.47
65	2.2	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	4	0.74
3	1.1	−	+	+	+	−	+	+	+	+	+	+	−	+	−	+	+	+	ND	0.5

*Bam HI amplicons were prepared from DNA from seven Amish pedigree lymphoblastoid cell cultures, GM05901 (driver), GM05987 (tester), GM05918, GM05961, GM05963, GM05993, GM05995 (columns A to G), five different placentas (columns H to L), three lymphoblastoid cell lines established from the biopsies of leukemic patients (columns M, N, and O), and two fibroblast cell cultures, DRL 484 and DRL 569, established from the biopsies of DMD patients (columns P and Q), transferred to GeneScreen membrane, and hybridized to the indicated probes. The plus sign means that the small Bam HI PARF allele was present in the sample (that is, the probe hybridized to a band of the correct size in the amplicon); the minus sign means that the small allele was not detected (see, for example, Fig. 3C). †The sizes of the alleles hybridizing to PARF's are indicated, where known. ‡The percentage of clones in the Bam HI difference product (obtained after three hybridization-amplification steps) that hybridized to the indicated clone. §Two different small alleles were found in the human population. ‖Two different large alleles were found in the human population. ND, not determined.

The probes for PARF's are not equally abundant in the difference product. To obtain a measure of this uneveness, we hybridized each cloned Bam HI PARF to a grid of 90 individual randomly picked clones from the difference product of the two siblings, and its frequency in the collection was determined (see percentage value in Table 2). Of 90 randomly picked elements, only 20 distinct polymorphic probes were present, and at very different frequencies. We estimate that there should be of the order of 1000 Bam HI PARF's between two individuals. The uneven distribution of PARF's in our difference products probably reflects the specific conditions used in our protocol, which was designed for the detection of a small number of differences between two nearly identical genomes. We found that, where probes for polymorphic loci were deliberately sought, more representative difference products could be generated by diminishing the number of rounds of hybridization and amplification, increasing the complexity of the representation or decreasing the total number of PCR cycles.

Applications to pathogen discovery. In principle, RDA can be used to isolate probes for pathogens when DNA from infected tissue is compared to DNA from uninfected tissue from the same individual. We have demonstrated this in the model case of the acquisition of a large (30 to 50 kbp) viral genome even with a single copy per cell. Probes for smaller viral genomes should also be detectable, but might require several applications of this procedure with several different restriction endonucleases in order to find fragments from the viral DNA that are readily amplified. As it is now described, RDA cannot reasonably be expected to detect probes for pathogens that are present at less than one copy per ten cells in infected tissue. The kinetic enrichment component would select against target sequences considerably when they are present at concentrations lower than other tester sequences. However, procedures for "normalization" (*18*), that equalize the concentrations of all tester sequences, could be applied prior to subtractive and kinetic enrichment.

Application of RDA to the discovery of pathogens requires precise matching of the polymorphisms from the infected and uninfected DNA sources. Tester and driver DNA can derive from the same individual, if the individual is not a genetic mosaic. These DNA's cannot derive from unrelated individuals, as the abundant polymorphic differences in their DNA's would obscure the detection of the pathogen. However, the uninfected DNA source (driver) could, in principle, come from an identical twin, or be the pooled DNA from the parents of the infected individual, because virtually every DNA restriction fragment found in the genomic DNA of the infected individual can be expected to be present in at least one parental DNA (*19*). Pooled parental drivers might be sought if the pathogen is suspected to be ubiquitous in the infected individual.

Applications to detecting genetic abnormalities in cancer. RDA should enable the discovery of genomic alterations occurring in cancer cells. These could be of two distinct types: those that result in loss of restriction endonuclease fragments, such as might occur from deletions or gene conversions extending over heterozygous polymorphisms, and those that produce new restriction endonuclease fragments, such as might result from genomic rearrangements. In the former case, RDA could be applied without modifications with DNA from cancer cells as driver and normal DNA as tester. Unfortunately, the presence of normal stroma in a cancer biopsy would certainly interfere with the detection of loss of genetic information in the cancer cell. Hence, either cultures of cancer cells, xenographs of tumors, or highly purified cancer cells obtained by physical separation would be needed as the source for tester.

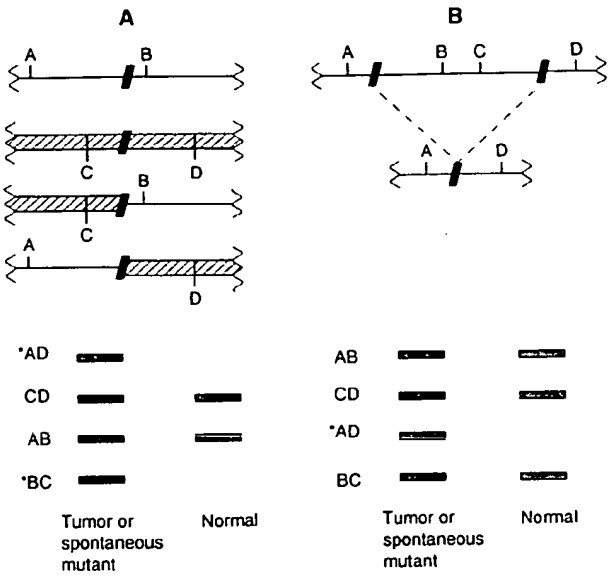


Fig. 4. Schematic representation of restriction endonuclease fragments of genomes that have suffered rearrangements. (**A**) Reciprocal translocation. (**B**) Deletion. Restriction endonuclease sites A, B, C, and D are as shown before and after rearrangements. Below is depicted the mobility of the new (*) and unrearranged fragments during gel electrophoresis. We assume that the normal chromosomes of the involved pair are present.

These restraints do not apply to the detection of genomic rearrangements. Genomic rearrangements, including translocations, insertions, inversions, and deletions, may result in the creation of new restriction endonuclease fragments "bridging" the site of the rearrangement (Fig. 4). Some of these bridging fragments may be amplifiable by PCR (fragment BC in Fig. 4A). Such bridging fragments would be discoverable by RDA when DNA from the tumor is used for preparation of tester amplicons and DNA from normal tissue of the same individual is used for preparation of driver amplicons.

The different-sized restriction endonuclease fragments created by genomic rearrangements can potentially be exploited another way. Fractionated size classes from tumor DNA digests may sometimes contain sequences that are not present in comparable size classes from normal DNA (Fig. 4). With the former as tester and the latter as driver, we can prepare amplicons after cleavage with a second restriction endonuclease and compare these in order to clone amplifiable restriction endonuclease fragments in proximity to the point of genetic rearrangement. The presence of normal cells among the tumor cells should not obscure the detection of probes for the rearrangement.

Application to genetic analysis. When RDA is applied to different individuals, and amplicons are used as the representation, probes for a special type of polymorphism that we call PARF's result. Other types of representations can yield different types of polymorphisms. In general, RDA may be useful for generating new sets of polymorphisms not only for species that have not previously undergone extensive molecular genetic characterization, but even for such well-studied species as humans and mice. Since PARF's are most often binary, they may be especially useful for creating a panel of probes that can be used with a standardized format for genetic typing.

RDA may find special uses when applied to pairwise comparisons of amplicons obtained from DNA's of individuals from specific groups. Straightforward application of RDA could yield probes for PARF's in the DNA of all individuals from a founder group affected by some autosomal dominant inherited disorder (the tester), but absent in the pooled DNA of all individuals from a normal group (the driver). Conversely, RDA could yield probes for PARF's present in the DNA of a normal individual (the tester), but absent in the DNA of all individuals from a founder group affected by a recessive inherited disorder (the driver). Combined with methods for coincidence cloning (*20*), such applications might accelerate the discovery of probes for rare PARF's in linkage disequilibrium with the dominant locus, or the absence of common PARF's in linkage disequilibrium with the recessive locus (*21*).

Many genetic diseases of progeny occur as a consequence of spontaneous germline genomic rearrangements in the gamete of one of the parents. The genome of the affected individual will in all probability have acquired restriction endonuclease fragments that are not present in the somatic cells of either parent. This situation is formally analogous to genetic rearrangements occurring in cancer cells (Fig. 4). In these special cases, RDA might be applied directly to the isolation of probes close to the sites of genetic abnormalities by taking DNA from the individual as tester and pooled DNA's from parents as driver.

Programmed rearrangements in somatic cells may also be discovered by RDA. Such events, for example, occur during the generation of diversity of the immune system (*1*), and have also been postulated during development of the nervous system (*22*). In principle, our method could be applied to the discovery of probes that detect these events. Finally, RDA may have special applications for the study of organisms that can be bred. Multiple backcrossing of a mutant strain to a polymorphic strain could lead to the identification of clonable restriction endonuclease polymorphic fragments tightly linked to the mutant locus.

Cautions. The data obtained with our protocol were highly reproducible; the same bands and even their relative intensities were obtained in multiple independent runs. Application of any new technique requires awareness of possible sources of artifact. One clear source can be PCR itself, because of the ease with which previous PCR products can contaminate reactions. Moreover, PCR products present after subtraction and enrichment do not necessarily reflect effective enrichment of target and may result from the stochastic nature of the process itself (*9*). Therefore each candidate difference product must be tested for its presence or absence in tester and driver amplicons. Another source of artifact can be anticipated during tissue sampling. Normal flora contaminating a specimen of tester will be readily enriched during difference analysis if that flora are not also present in driver. Other sources of artifact can be readily imagined. For example, we do not know to what extent we may find programmed genetic rearrangements specific for certain tissues or organs. We also do not know the extent of genetic mosaicism in somatic tissues, the result of spontaneous genetic events occurring during early development. Even so, application of RDA should provide an economical route to solving many types of genetic problems.

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7. Throughout this article, we use "target" to refer to sequences present in one population, the "tester," but absent in another, the "driver."
8. In general, a single cycle of subtraction can be expected to yield enrichments of target of the order of fN where N is the molar excess of driver to tester and f is the fraction of driver that reanneals.
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10. To visualize this, consider viral sequences pres-

ent in excess (ten times more) relative to single-copy β-globin sequences. At early stages of self-reannealing, when 5.0 percent of the viral sequences are reannealed, only 0.5 percent of the β-globin sequences will be reannealed. The ratio of the viral sequences to the β-globin sequences in the double-stranded DNA will then be 5 percent of 10 to 0.5 percent of 1 (one hundred times more).

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12. For preparation of amplicons both tester and driver DNA samples were digested with restriction endonuclease (New England Biolabs) and 1 μg of each DNA digest was mixed with 0.5 nmol of 24-bp and of 12-bp unphosphorylated oligonucleotides (Table 1, primer set 1) in 30 μl of T4 DNA ligase buffer (New England Biolabs). Oligonucleotides were annealed by cooling the mixture gradually from 50° to 10°C for 1 hour and then ligated to human DNA fragments by overnight incubation with 400 U of T4 DNA ligase at 16°C. After ligation, both tester and driver DNA samples were amplified. Each of ten tubes taken for preparation of driver amplicons and two tubes used for preparation of tester amplicons contained (in 400 μl) 67 mM tris-HCl, pH 8.8 at 25°C, 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, bovine serum albumin (100 μg/ml), 300 μM (each) dATP, dGTP, dCTP, and dTTP, 1 μM 24-bp primer, and 80 ng of DNA with ligated adaptors. The tubes were incubated for 3 minutes at 72°C in a thermal cycler (Perkin-Elmer Cetus), 15 U of Taq polymerase (AmpliTaq, Perkin-Elmer Cetus) was added, the reactions were overlaid with mineral oil and incubated for 5 minutes to fill in 5′ protruding ends of ligated adaptors, and amplified for 20 cycles (each cycle including 1 minute incubation at 95°C and 3 minutes at 72°C, with the last cycle followed by an extension at 72°C for 10 minutes). After amplification both driver and tester amplicons were digested with the same restriction endonuclease (10 U/μg) to cleave away adaptors. For removal of adaptors, 10 μg of tester amplicon DNA digest was subjected to electrophoresis through 2 percent NuSieve agarose (low melting point, FMC BioProducts), and DNA fragments (150 to 1500 bp) were recovered after melting of the agarose slice and Qiagen-tip20 chromatography (Quiagen Inc.).
13. These estimates in degree of simplification were calculated from data on the mean restriction endonuclease fragment lengths and the proportion of fragments of <1 kbp [D. T. Bishop, J. A. Williamson, M. H. Skolnick, *Am. J. Hum. Genet.* 35, 795 (1983)]. Our calculations of the number of fragments with an amplifiable size are not based on complete information. We do not know, for example, the exact mathematical dependence of amplification efficiency upon fragment length, nor the proportion of fragments of any length that amplify poorly. Moreover, the occurrence of restriction endonuclease cleavage sites is not truly random.
14. In preparation for the hybridization and amplification step, fragments of tester amplicons were ligated to new adaptors (Table 1, primer set 2, and Fig. 1). The tester amplicon (0.5 μg) ligated to adaptors and the driver amplicon (40 μg) DNA's were mixed, ethanol-precipitated, dissolved in 4 μl of 3 × EE buffer (*15*), overlaid with 30 μl of mineral oil (Perkin-Elmer Cetus), and denatured by heat; 1 μl of 5 M NaCl solution was added and DNA was hybridized for 20 hours at 67°C. At the end of hybridization, part (10 percent) of the resulting DNA was incubated with 15 U of Taq polymerase (5 minutes, 72°C) in 400 μl of PCR mixture without primer to fill in ends of the reannealed tester, and then amplified for 10 cycles (1 minute at 95°C, 3 minutes at 70°C, and held for 10 minutes more for the last round) after addition of the same 24-bp oligonucleotide to which the tester was ligated. Single-stranded DNA molecules present after amplification were degraded by a 30-minute incubation with 20 U of mung bean nuclease (New England Biolabs) in a volume 40 μl, diluted (1:5) in 50 mM tris-HCl (pH 8.9), and heated (95°C, 5 minutes) to inactivate the enzyme. A portion (40 μl) of the solution was amplified for 15 to 20 cycles under the same conditions as before the mung bean nuclease treatment. Amplified DNA (3 to 5 μg) was digested with the original restriction endonuclease, and 200 ng of the digest was ligated to the third adaptor (Table 1, primer set 3). This DNA (50 to 100 ng) was mixed with 40 μg of driver amplicon and the hybridization and amplification procedures were repeated as in the first cycle. A sample (200 ng) of the digest obtained after the second hybridization-amplification step (Table 1, primer set 2) was then ligated to the second set of adaptors, and 100 to 400 pg of this material together with 40 μg of driver amplicon was taken for the third round of hybridization, with a final amplification after mung bean nuclease digestion for 20 to 25 cycles. When a fourth hybridization-amplification step was performed, 5 pg of material from the third round was ligated to the third adaptors (Table 1, primer set 3) and mixed with 40 μg of driver amplicon before proceeding.
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16. PCR adaptors were alternated between rounds of hybridization and amplification to avoid the accumulation of PCR products that might interfere with subsequent amplifications. The adaptors are designed to reconstruct the original restriction endonuclease cleavage sites that defined the tester fragments, and are therefore removable by cleavage with that same enzyme.
17. If enriched tester is insufficiently diluted, further enrichment due to the subtractive component will be hampered because driver amplicon sequences may not then be in excess over amplicon sequences from tester. Further enrichment due to the kinetic component will also be diminished because of the excessive self-reannealing of non-target tester. If enriched tester is diluted too much, so little target will reanneal that it may no longer be detectable by PCR. The protocols described should serve as a practical guide.
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19. Homologous germline recombination between two polymorphic restriction endonuclease sites would create a new restriction fragment length polymorphism present only in the progeny, but this should be a rare event.
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21. We do not have enough experience with RDA to predict what would happen if DNA's pooled from individuals of one group were used as tester and DNA's pooled from individuals of another group were used as driver. We expect the difference product would yield probes to PARF's common to most of the first but absent in all of the second group.
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